Urea biosensor based on amperometric pH-sensing with hematein as a pH-sensitive redox mediator

Andrea Pizzariello [a,*], Miroslav Stredanský [a], Silvia Stredanská [a], Stainslav Miertuš [b]

[a] *POLYtech, Area Science Park, Padriciano 99, 34122 Trieste, Italy*
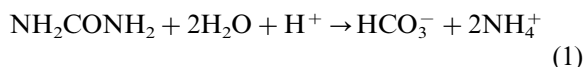[b] *ICS-UNIDO, Area Science Park, Padriciano 99, 34012 Trieste, Italy*

Abstract

The natural dye hematein in water solution was used as a pH-sensitive redox-active mediator for amperometric pH-sensing. The electrochemical characteristics were studied using cyclic voltammetry and chronoamperometry. Several types of urea biosensors were constructed with urease on the surface of platinum and graphite composite electrodes or in the bulk of the graphite composite. They were used for the amperometric urea determination at a working potential of 0 mV (versus SCE) using 0.5 mM hematein. Detection limits and response linearity was in the micromolar range depending on the biosensor type, concentration and pH of buffers used. An interference study of various cations, anions, and substances, which may be present in real samples demonstrated good selectivity for the determination of urea. The biosensors showed good operational (> 3 h) and storage (> 3 months) stability. The results of urea determination in blood and urine obtained by biosensor correlated well with those obtained by a spectrophotometric reference method. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Urea is widely distributed in nature and its analysis is of considerable interest in clinical chemistry, agro-food chemistry, and environmental monitoring.

Many methods of urea determination are based on its hydrolysis by urease and successive measurement of ions consumed or produced:

$$NH_2CONH_2 + 2H_2O + H^+ \rightarrow HCO_3^- + 2NH_4^+ \tag{1}$$

The assay with urease can be followed by various means, including calorimetric [1,2], fluorimetric [3], and electrochemical [4–6] techniques. The possibility of electrochemical detection led to the development of a large number of urea biosensors based on conductometric [7,8], potentiometric

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* Corresponding author. Tel.: + 39-040-3756621; fax: + 39-040-9220016.

E-mail address: pizzarie@polytech3.area.trieste.it (A. Pizzariello).

[4,9–12], and amperometric [6,13–17] transducers. The potentiometric transducers with the application of pH-sensitive electrodes, ion-selective electrodes and ion-sensitive field effect transistors are the most frequently used for the urea biosensors. However, they suffer from the inherent characteristics of potentiometry, i.e. rather slow responses, drift of the base line, vulnerability to the interference of other ions in sample solution, and relatively high detection limits.

On the other hand, amperometric urea biosensors showed a better sensitivity and lower detection limits. Osakai et al. [13] described an amperometric urea biosensor based on ammonium-ion selective gel electrode. Systems with a coupled enzyme, e.g. glutamate dehydrogenase [14,15] or with the application of polymer-modified electrodes [6,16] were developed. However, most amperometric urea sensors utilise the sensing ammonium ion, which could interfere with the analysis of real samples. Kirstein et al. [17] developed an amperometric sensor for urea using the dependence of the electrochemical hydrazine oxidation on concentration of OH^-. Disadvantages are in a low sensitivity (linear range from 0.8 to 35 mM) and in the use of such a hazardous compound as hydrazine. Recently, we presented a concept of amperometric pH-sensing biosensors based on the use of pH-sensitive redox-active mediators [18,19].

In this paper, an amperometric urea biosensor exploiting hematein as the pH-sensitive redox-active mediator is described. The characteristics and performance of various concepts of the sensor based on platinum and solid composite graphite electrodes are evaluated. Determination of urea in blood and urine are also presented.

2. Experimental

2.1. Materials

Urease (3.5.1.5; from jack beans, type VII) and *n*-eicosane were purchased from Sigma (St. Louis, MO). Hematein, graphite powder and other reagents of analytical purity were from Fluka (Buchs, Switzerland).

2.2. Preparation of enzyme electrodes

The solid composite graphite electrodes were prepared as described earlier [20,21]. About 50 mg of the graphite powder was added in 50 mg of melted *n*-eicosane at 45°C and mixed intensively. The melted mixture was used to fill a hole (2.0 mm in diameter, 3 mm in depth) at the end of an electrode (a PVC tip, 2 mm i.d., 5 mm o.d., length 20 mm). After cooling at room temperature the excess of solidified materials was removed with the aid of sand paper. The surface was then polished on a sheet of paper. Electrical contact was ensured by a brass rod. Urease was applied in the form of solution (1 µl; 4.4 U µl^{-1}) spread over the electrode surface. After drying at room temperature the surface was covered with a dialysis membrane (Spectra/Por type 1, Spectrum Medical Industries, TX) fixed by a thin PVC O-ring.

For the preparation of a bulk-modified urea biosensor, the graphite was first mixed with enzyme [22]. The enzyme (1.5% with regard to the final weight of the composite material) was dissolved in water and graphite was added. Then the suspension was mixed thoroughly until water was evaporated. The traces of water were eliminated by drying in dessicator overnight. The preparation obtained was mixed with the melted *n*-eicosane (50% with regard to the final weight of the composite material) and packed in the same way as described above.

Platinum electrode (2 mm in diameter, AMEL) was carefully cleaned by polishing to a mirror finish with an alumina slurry of 1 µm and sonicated in pure water to remove the embedded alumina particles. Then 1 µl of urease solution (4.4 U µl^{-1}) was spread over the electrode surface and after drying at room temperature the surface was covered with a dialysis membrane fixed by a rubber O-ring.

2.3. Apparatus and measurements

Cyclic voltammetric studies were carried out with the computerised electrochemical analyser AMEL 433/W (Milan, Italy). A saturated calomel

electrode (SCE) and a Pt spherical electrode were used as the reference and the counter electrodes, respectively.

Chronoamperometric studies were performed with potentiostat (AMEL 559) and a x-t recorder (AMEL 868). Experiments were carried out in batch-mode at 25°C in a jacketed reaction vessel equipped with magnetic stirring. For the pH-dependency curve measurements, the working electrodes without enzymes and the reference electrode (SCE) were immersed in 10 ml of 0.05 M phosphate solution (pH about 10) containing 0.5 mM hematein and polarised to select constant operating potentials. When the signal output was stabilised the current changes were monitored after each addition of the 2 M HCl solution. Simultaneously, the pH values were measured by the pH-meter (PHM 85 precision pH-meter, Radiometer, Copenhagen, Denmark).

For biosensor measurements, the electrodes with enzymes and the reference electrode (SCE) were immersed in 10 ml of 1 mM phosphate buffer containing 0.1 M electrolyte (NaCl) and 0.5 mM hematein. After the current stabilisation a standard urea solution was added, and current-time response curves were recorded. The height of the recorded wave was correlated to the concentration of the analyte. Blood and urine samples were collected from healthy persons before breakfast. Each sample was divided into different aliquots. Part of these aliquots was analysed for urea by a standard spectrophotometric method and the others using the biosensor systems reported here. In real sample analysis 20 μl of blood and 10 μl of 10-times diluted urine were applied when the Pt-based and surface-modified graphite biosensors were employed. 50 μl of blood and 25 μl of 10-times diluted urine were applied when the bulk-modified graphite biosensors were employed. The amount of urea was determined from standard calibration curves.

A Standard spectrophotometric method for urea determination based on the Berthelot reaction [23] was used as a reference. The absorbance of the indophenol produced was measured at 550 nm.

3. Results and discussion

3.1. Effect of pH on hematein electrochemical properties

Hematein is a natural dye used in selective staining of biologic materials [24]. It is a stable water-soluble electroactive compound usable also as a pH indicator with turning from yellow at acidic pH to pink violet at basic pH. Cyclic voltammograms of hematein measured with the composite graphite electrode at various pH values demonstrate its strong pH-dependence (Fig. 1). Both the anodic and cathodic peaks shift to more positive potentials as pH decreased. The shift in the cathodic peak potential is 37 mV per pH unit. It is also expected, that at the constant working potential the pH change is accompanied with a current change. The current changes at two selected potentials (200 and 0 mV versus SCE) are illustrated in Fig. 2. The use of the working potential of 200 mV (versus SCE) seems to be more advantageous, because of the wider range of sensitivity to pH, however, a slow electropolymerisation of hematein occurred at pH higher than 6.5, which caused a signal drift affecting the measurement precision. On the other hand using the working potential of 0 mV (versus SCE) the hematein electropolymerisation was observed only at pH higher than 8.4. Thus, the working potential of 0 mV (versus SCE) was selected for further experiments. The use of this potential is also favourable due to lower electrochemical interferences of real samples.

The behaviour of hematein on Pt-electrode was reported earlier [18,19]. It is interesting, that with the Pt-electrode the interval for monitoring the possible pH-change at 0 mV (versus SCE) was wider (4–10) than with the composite electrode.

3.2. Urea biosensors with urease on the transducer surface

The urea biosensor was easily prepared by spreading urease on the electrode surface subsequently covered with a dialysis membrane. When the biosensor is placed in the buffer containing hematein and polarised at 0 mV (versus SCE) the
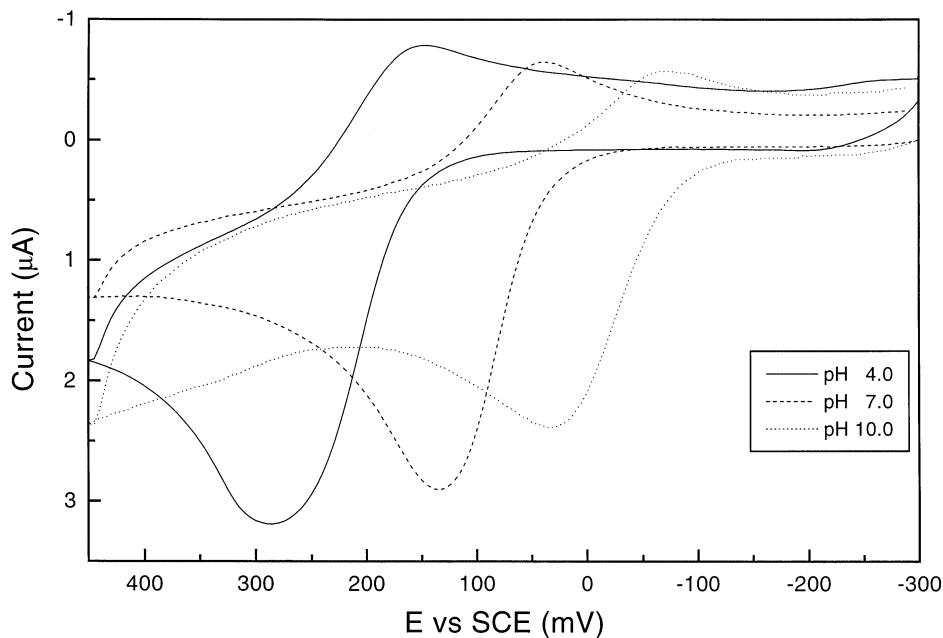
Fig. 1. Cyclic voltammograms of hematein on a *n*-eicosane–graphite composite electrode obtained at various pH in 0.1 M phosphate solution. Scan rate: 50 mV s^{-1}.

current signal is stabilised after 2–3 min. The addition of urea leads to a local pH-change in the electrode surface vicinity that is recorded as a current change.

A typical calibration curve of the Pt-based urea sensor is illustrated in Fig. 3., whereas Fig. 3, inset (a), shows the typical urea response curve (current versus time) for the same sensor. The response was linear in range from 10 to 250 μM and the detection limit was 3 μM ($S/N = 3$). The sensitivity in the linear region was 1.95 μ AmM^{-1} cm^{-2} and the response was about 2 min. To assess reproducibility, six sequential measurements of 20 μM were performed, which gave a relative standard deviation (s_r) of 4.3%. These data are fully comparable with those reported for other amperometric urea biosensors [6,13–17]. The sensor sensitivity can be even improved with increasing hematein concentration. The increase of hematein concentration from 0.5 to 1.5 mM doubled the sensor sensitivity, but the measurement reproducibility was affected negatively ($s_r = 7.6$; $n = 6$). A further increase of hematein concentration is, however, difficult due to its limited solubility in water.

On contrary to the Pt-based biosensor, whose linearity was affected by the initial pH only slightly in the range from 6.0 to 7.5, the linearity of the graphite composite urea biosensor strongly depended on this parameter (Fig. 4). The response was linear in the range from 60 to 300 μM (pH
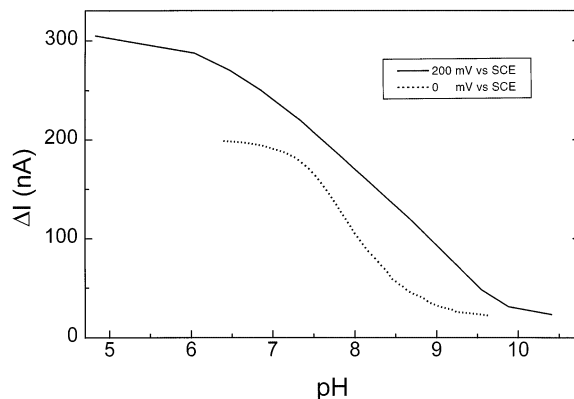

Fig. 2. Dependence of the current change of pH of a *n*-eicosane–graphite composite electrode immersed in 0.5 mM hematein solution. Applied potential: 200 mV (solid line) and 0 mV (doted line) versus SCE.
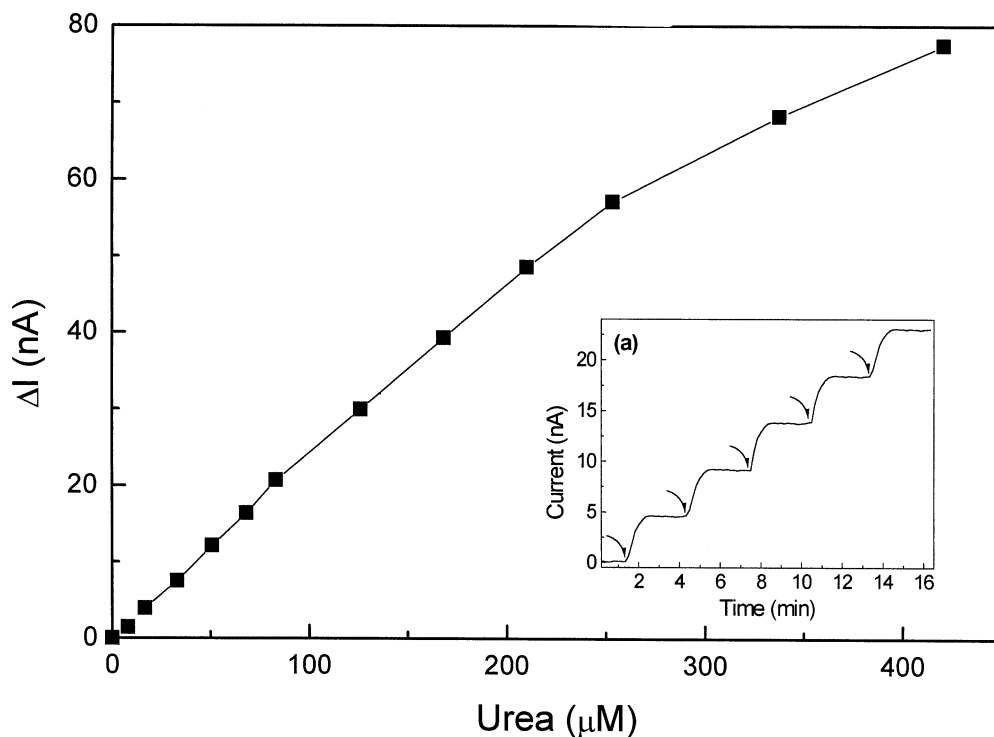
Fig. 3. Dependence of the current change on urea concentration of the biosensor based on platinum electrode with urease layer. Inset (a): electrode response to successive additions of urea. Arrows show the successive addition of 20 μM urea. Conditions: working potential 0 mV versus SCE, 1 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl and 0.5 mM hematein.

6.9), from 20 to 170 μM (pH 7.3), and from 5 to 100 μM (pH 7.7). The effect of the initial pH value is clearly connected with the redox properties of hematein on the composite electrode surface at various pH (Section 3.1, Fig. 2). In fact, different slope currents were observed, namely 6.9, 68.1, 113.5 nA per pH unit at 6.9, 7.2 and 7.7 as initial pH values, respectively. Moreover, the stronger curvature of the 7.7 pH calibration curve at high substrate concentrations can be explained by a decrease of enzymatic reaction rate owing the local pH values exceeding the pH optimum (pH 7.0–7.4) [17]. On the other hand, the sensitivity of these biosensors in the linear region was independent of the initial pH (0.98 ± 0.02 μ AmM^{-1} cm^{-2} at the mentioned pH values). Although the graphite composite biosensors exhibit inferior analytical characteristics than Pt-based ones, they are still suitable for the urea determination in real samples, such as urine, blood, milk, fertilisers,

fermentation media, because of their low detection limit. Moreover, the use of the composite transducers allows a cheap mass production of biosensors. In fact, the transducers based on n-eicosane and other solid binding matrices (SBMs) have been used in the industrial production of amperometric biosensors for glucose, fructose, ethanol, L-lactate, and L-malate [21,25].

3.3. Urea biosensors with urease incorporated in the composite electrode bulk

The incorporation of the biocatalyst within the bulk of a composite matrix offers the possibility to obtain a renewable biosensors, a high stability of the incorporated biocatalyst, and the ease and low cost of fabrication [21,26]. Typical calibrations of the bulk type urea sensors are illustrated in Fig. 5. The response was linear in a wider range, from 25 to 350 μM, in comparison to that

obtained with the surface type biosensor in the corresponding measuring buffer (pH 7.3; 1 mM phosphate), but the sensitivity in the linear was lower (0.199 μA mM^{-1} cm^{-2}). A change in the electrode conducting component/mediator kinetics [27,28], a different proximity of urease of the transducting element modifying the diffusion path of enzymatic products [29] and a different abundance of active biocatalysts interfaced to the electrode surface itself [30] are involved in the decreasing sensitivity observed for the urea sensors investigated. As expected, the response of the electrode was affected also by a buffer concentration due to an alteration of its buffering capacity. In fact, it is well known that for pH based sensors the response-sensitivity and linear range is dependent on the buffer used and its buffering capacity [31]. So, the application of a more diluted buffer (1 mM) resulted in better biosensor sensitivity, though a more noisy signal was recorded. The

application of a more concentrated buffer (5 mM) decreased the sensor sensitivity (0.064 μA mM^{-1} cm^{-2}), but the linear region was significantly extended (50 $\div$ 1000 μM) and the signal noise was minimised, besides it could be suitable for urea determination in undiluted samples.

One of the major problems of composite biosensors is the insufficient reproducibility in their fabrication, because it is difficult to obtain homogeneous distribution of all components [20,26]. The estimation of a fabrication reproducibility of urea biosensors was based on statistical evaluation of the sensitivity values. The s_r of the values of bulk type urea sensors was 13.5% ($n = 7$), which was higher than found for the surface type (6.5%, $n = 7$), but comparable with that earlier reported for SBM-based biosensors [20]. However, the use of a urea standard for measuring real samples eliminated the problems of insufficient reproducibility in biosensor fabrication.
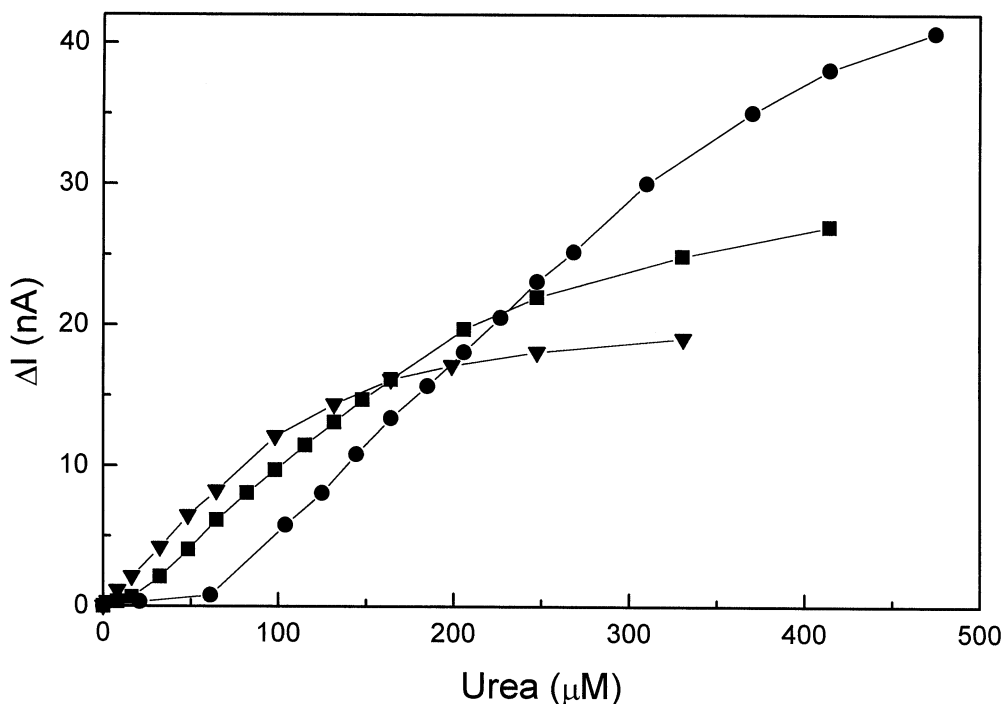

Fig. 4. Dependence of the current change on urea concentration of the biosensor based on *n*-eicosane–graphite composite electrode with urease layer at pH 6.9 (●), 7.3 (■), 7.7 (▼). Conditions: working potential 0 mV versus SCE, 1 mM phosphate buffer containing 0.1 M NaCl and 0.5 mM hematein.
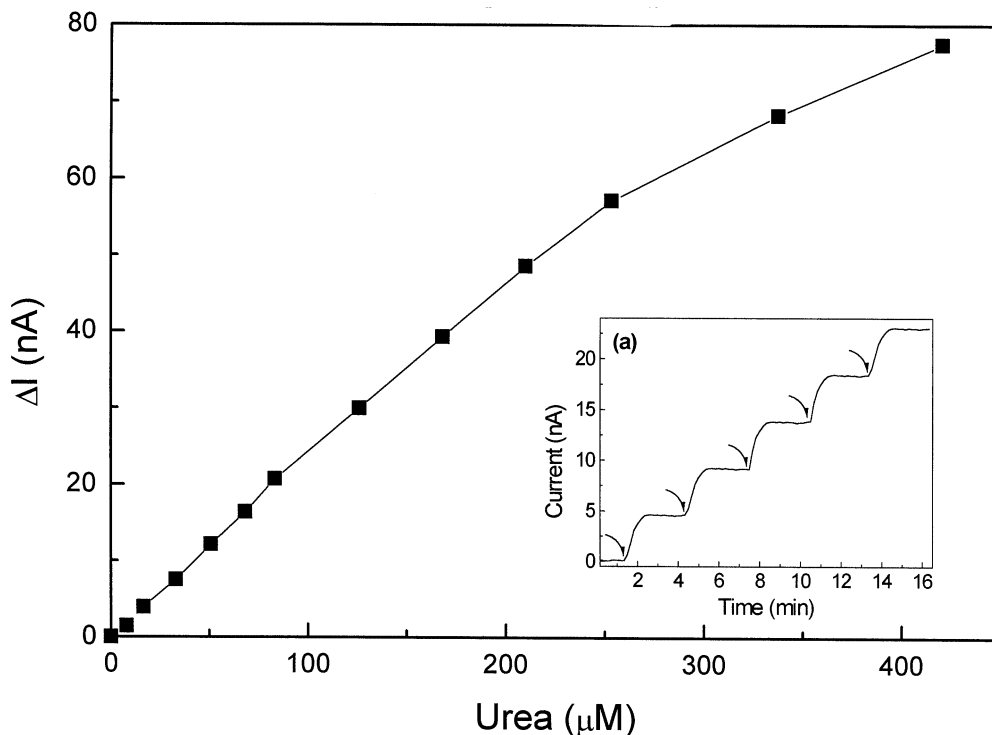
Fig. 5. Dependence of the current change on urea concentration of the biosensor based on *n*-eicosane–graphite composite electrode bulk-modified with urease at phosphate buffer concentration of 1 mM (●) or 5 mM (■). Conditions: working potential 0 mV versus SCE, the buffer (pH 7.3) containing 0.1 M NaCl and 0.5 mM hematein.

3.4. Interferences

Interferences of cations, anions and other potential interfering compounds on the response of the presented biosensors to 0.1 mM urea were studied. As all measurements were performed in phosphate buffer containing also 0.1 M NaCl (as supporting electrolyte), the interferences from Na^+ and Cl^- are excluded. The anions (sulphate, carbonate, L-lactate, pyruvate) and cations (K^+, Ca^{2+}, Mg^{2+}) did not interfere in concentration up to 10 mM. The same is valid also for glucose, fructose, sucrose, lactose and ethanol. Ammonium and bicarbonate ions, which are products of urea hydrolysis, did not show any interference up to 1 mM. Most of amperometric and potentiometric urea biosensors are based on NH_4^+ monitoring [4,6,9,10,12–16]. As NH_4^+ often occurs in real samples, it causes a serious interference. The insensitivity of the presented biosensors to NH_4^+ overcomes this.

Among the other possible interfering compounds, urate did not affect the response to urea in concentration up to 0.1 mM, but ascorbate increased the measured current due to its oxidation on the electrode surface. This increase was in the range from 25 to 60% for various sensor types when the ascorbate concentration was equimolar to urea (0.1 mM). Taking into account, that ascorbate is present in real samples (urine, blood, milk, etc.) at much lower concentration than urea [15], thus its interference is much less significant. Several reports have indicated that numerous phenolic species appears to possess a metallic ions binding properties [32–37]. So, we have investigated the effects of different metallic ions on the response to urea. Solutions containing 0.1 mM urea with the addition of 40 μM each of Fe^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+} were analysed with the presented biosensors. Compared with the results obtained with the current response of 0.1

mM standard urea solution, no appreciable change of the current was found for solutions with added metallic ions.

3.5. Operational and storage stability

The operational stability of all types of the urea biosensors presented here was satisfactory. The sensitivity of the sensors did not change after 3 h of discontinuous use. A repeated dry and use of the biosensors (at least three times) did not show any effect, either.

The presented biosensors display excellent storage characteristics when stored dry in dessicator at room temperature. The Pt-based biosensors were stable at least 3 weeks without any loss in their sensitivity. The surface-modified composite biosensors remained stable 3 months. After 6 months $71.3 \pm 6.5\%$ ($n = 5$) of the initial sensitivity was found. The unrenewed electrode surface of the bulk-modified biosensors gave a gradually decaying response, by approximately 40% at the end of the 6 months, but after renewing (mechanical polishing) of the active surface the initial sensitivity was restored almost completely (93.7 ± 11.6; $n = 5$). The extended room-temperature stability of the urease are better than earlier achievements in the field of biosensors for urea determination [4–17], where the enzyme electrodes were stored at lower temperatures and in wet status. The found stability is comparable with other amperometric biosensors based on SBMs [20–22,25], as also resulting from several earlier studies [38–40], that testify a remarkable resistance to thermal denaturation of enzymes immobilised within hydrophobic semi-solid matrices.

3.6. Application of the biosensors in biological samples

To demonstrate the feasibility of the biosensors for urea analysis, samples of fresh blood and urine from healthy persons were analysed. The analyses were performed without any sample pretreatment and the results were compared with those obtained with the standard spectrophotometric method [23]. The values of urea concentration in blood obtained with biosensors were higher than those obtained with the standard method. The overvalues were about 10% for the surface-modified composite sensors and in the range from 25 to 50% for the Pt-based and bulk-type ones. Therefore, postdifferential measurements were used, i.e. the content of urea in blood determined by the urea sensor was corrected by the response of the sensor where urease was replaced by bovine serum albumin (BSA) (blank electrode). In this case, a good agreement of the urea contents in blood with the spectrophotometric data was obtained (Table 1). The interferences were probably due to electroactive species of plasma, but some interaction of blood corpuscles and other components with hematein and electrode surface can not be excluded. On the other hand urea analyses in urine with the biosensors agreed well with the spectrophotometric data without the support of the postdifferential method (Table 1). The dynamics of the blood interference phenomenon and the application of the presented biosensors to the urea determination in other real samples, such as milk and fermentation media, are being investigated in our laboratory.

Table 1
Urea analyses in blood and urine[a]

Sample	Urea concentration (g 1^{-1})			
	Pt-based biosensor	Surface type composite biosensor	Bulk type composite biosensor	Reference method
Blood 1	0.283 ± 0.012[b]	0.263 ± 0.011[b]	0.266 ± 0.013[b]	0.273 ± 0.015
Blood 2	0.173 ± 0.007[b]	0.186 ± 0.006[b]	0.192 ± 0.008[b]	0.181 ± 0.010
Urine 1	17.1 ± 0.7	16.0 ± 0.6	15.6 ± 0.7	16.4 ± 0.8
Urine 2	13.8 ± 0.5	14.1 ± 0.4	14.8 ± 0.6	14.2 ± 0.7

[a] Comparison between results obtained with the biosensors and a reference spectrophotometric procedure ($n = 5$).
[b] Results obtained by post-differential measurement.

4. Conclusion

Most urea biosensors are based on potentiometric mode of detection. However, a high detection limit [4,12], a non constant sensitivity if variable amounts of interfering ions are present in samples and standards [4,9–12] and a short lifetime [4,11] critically affect the performance profile of those biosensors. On the other hand, several authors have reported amperometric approaches for the construction of a reliable urea biosensors, but these systems equally show important drawbacks, such as high working potential [14], susceptibility to the amount of interfering ions [16], poor lifetime [6,13–15] and high detection limit [14–17]. Compared with the above mentioned amperometric urea biosensors, our detection mechanisms are different and involve the use of amperometric pH-sensing with hematein as a pH-sensitive redox probe molecule. The urea biosensors based on platinum and composite graphite electrodes showed a good sensitivity, stability and specificity. Their responses to urea were linear in micromolar ranges. Their signal was not affected by ammonium and other ions present in biological samples. Particularly SBM-based composite transducer has proven to be an excellent medium for the immobilisation of active urease in suitable quantities to produce a sensitive biosensor electrode. They were applied to the determination of urea in blood and urine and the results were comparable to those obtained by the reference method. The presented biosensors could find a practical utilisation, because of their satisfactory analytical characteristics, simple and cheap fabrication, and the possibility of miniaturisation and mass production.

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